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Sox9 is required for cartilage formation

Weimin Bi, Jian Min Deng, Zhaoping Zhang, Richard R. Behringer & Benoit de Crombrugghe

Chondrogenesis results in the formation of cartilages, initial skeletal elements that can serve as templates for endochondral bone formation. Cartilage formation begins with the condensation of mesenchyme cells followed by their differentiation into chondrocytes. Although much is known about the terminal differentiation products that are expressed by chondrocytes[1–3], little is known about the factors that specify the chondrocyte lineage[4–6]. SOX9 is a high-mobility-group (HMG) domain transcription factor that is expressed in chondrocytes and other tissues[7–12]. In humans, *SOX9* haploinsufficiency results in campomelic dysplasia, a lethal skeletal malformation syndrome, and XY sex reversal[7,13–16]. During embryogenesis, *Sox9* is expressed in all cartilage primordia and cartilages, coincident with the expression of the collagen α1(II) gene (*Col2a1*; refs 8,11,12). *Sox9* is also expressed in other tissues, including the central nervous and urogenital systems[8–12]. Sox9 binds to essential sequences in the *Col2a1* and collagen α2(XI) gene (*Col11a2*) chondrocyte-specific enhancers and can activate these enhancers in non-chondrocytic cells[17–19]. Here, *Sox9* is identified as a regulator of the chondrocyte lineage. In mouse chimaeras, *Sox9*$^{-/-}$ cells are excluded from all cartilages but are present as a juxtaposed mesenchyme that does not express the chondrocyte-specific markers *Col2a1*, *Col9a2*, *Col11a2* and *Agc*. This exclusion occurred cell autonomously at the condensing mesenchyme stage of chondrogenesis. Moreover, no cartilage developed in teratomas derived from *Sox9*$^{-/-}$ embryonic stem (ES) cells. Our results identify Sox9 as the first transcription factor that is essential for chondrocyte differentiation and cartilage formation.

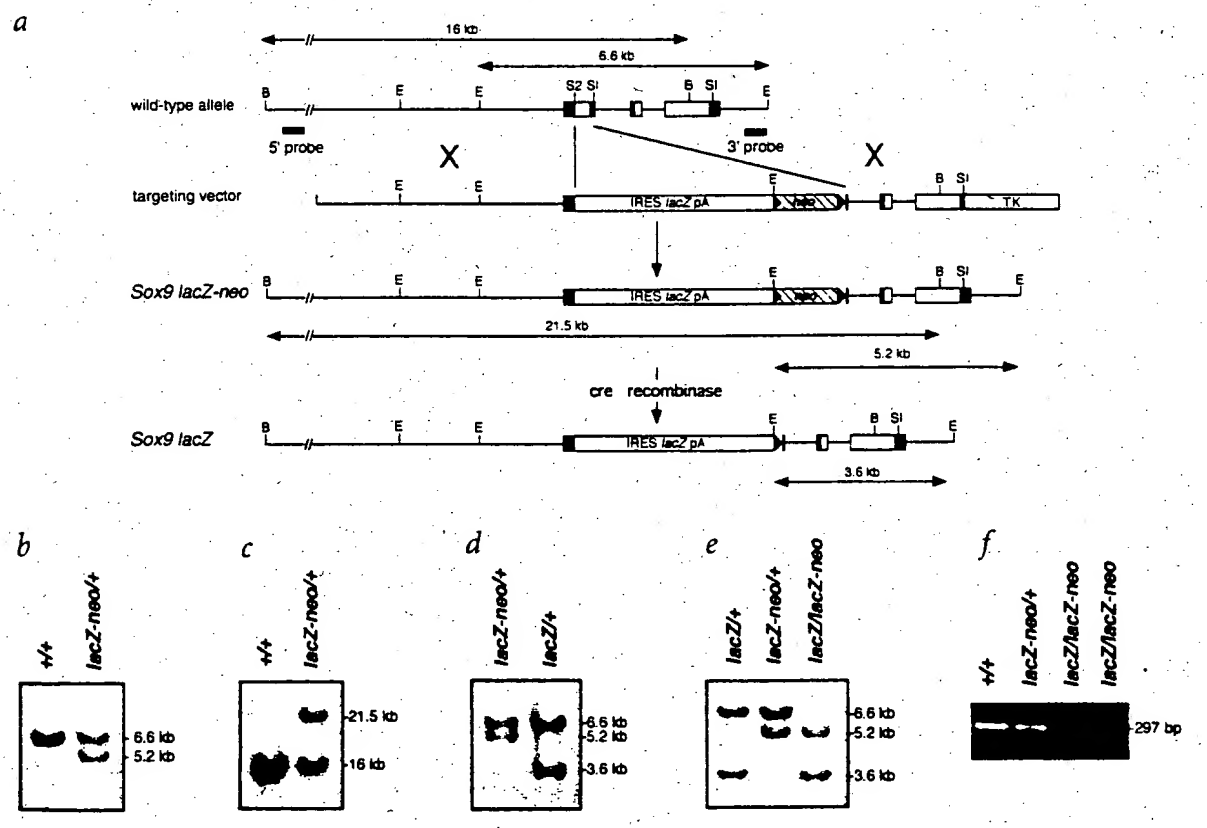

Fig. 1 Generation of *Sox9*-mutant ES cell lines. *a*, Strategy to introduce *lacZ* into the *Sox9* locus in ES cells. The three coding exons of *Sox9* are shown. Open boxes, exons; shaded boxes, 5′ and 3′ UTR; filled boxes, HMG box. The 450-bp *Sac*II–*Sac*I region containing the translation start codon and the 5′ region of the HMG box of *Sox9* was replaced by the IRES-*lacZ*-pA-lox*P*-flanked PGKneobpA cassette. E, *Eco*RI; B, *Bgl*II; SI, *Sac*I; S2, *Sac*II; neo, PGKneobpA; triangles, *loxP* sites; TK, MC1tkpA. Transient cre recombinase expression removes the *loxP*-flanked neo cassette from the targeted locus. Once the neo cassette was removed, the remaining wild-type allele was mutated by the original targeting vector. The predicted *Bgl*II and *Eco*RI fragments for wild-type and targeted alleles and the 5′ and 3′ Southern probes are indicated. *b*–*e*, Southern analysis of targeted ES cell lines. The sizes of the hybridizing restriction fragments are indicated, as well as the initial targeting of the *Sox9* locus in wild-type (+/+) ES cells to generate *Sox9 lacZ-neo*/+ ES cell lines. *b*, *Eco*RI digestion and hybridization with the 3′ external probe. *c*, *Bgl*II digestion and hybridization with the 5′ external probe. *d*, Removal of the neo cassette from the *lacZ-neo* allele by cre recombinase to generate *Sox9 lacZ*/+ ES cell lines (*Eco*RI digestion and hybridization with the 3′ external probe). *e*, Targeting of the remaining *Sox9* wild-type allele in *Sox9 lacZ*/+ ES cell lines to generate *Sox9 lacZ*/*lacZ-neo* ES cell lines. It is also possible to retarget the *Sox9 lacZ* allele to regenerate *Sox9 lacZ-neo*/+ ES cell lines (*Eco*RI digestion and hybridization with the 3′ external probe). *f*, PCR analysis to confirm the *Sox9* deletion. Primers located in the deleted region were used to amplify a 297-bp *Sox9* fragment. No amplified product is generated from two independently derived *Sox9 lacZ*/*lacZ-neo* ES cell lines.

Department of Molecular Genetics, The University of Texas, M.D. Anderson Cancer Center, Houston, Texas 77030, USA. Correspondence should be addressed to B. de C. (e-mail: benoit_decrombrugghe@molgen.mdacc.tmc.edu) or R.R.B. (e-mail: bhr@molgen.mdacc.tmc.edu).

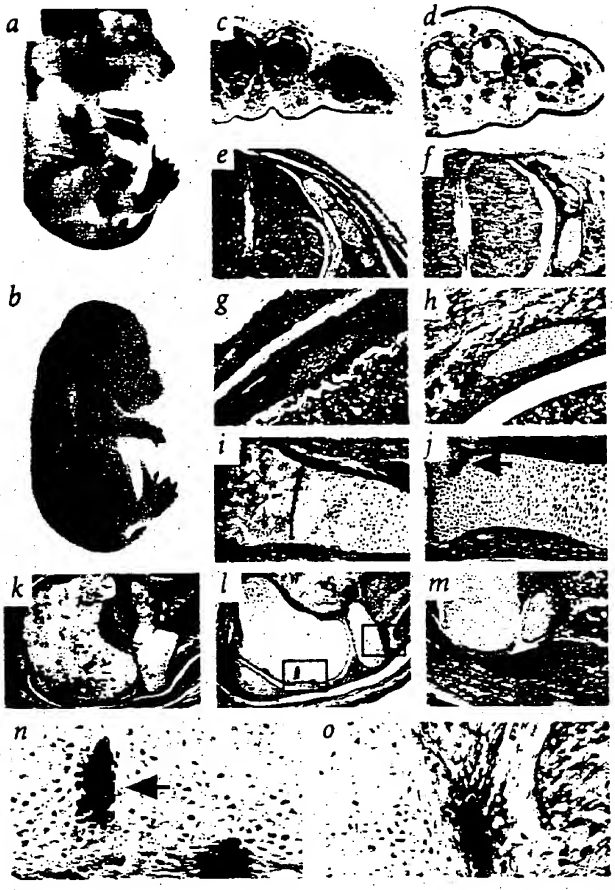

Fig. 2 *Sox9* chimaeras at 15.5 dpc. Whole-mount X-gal stained chimaeric embryos derived by injection of $Sox9^{+/-}$ (*a*) and $Sox9^{-/-}$ (*b*) ES cells into wild-type blastocysts. Histological analysis of $Sox9^{+/-}$ chimaeras showed that $Sox9^{+/-}$ β-gal–positive (blue) cells were located in the cartilages, intermingled with wild-type (pink) cells in the digits (*c*), vertebrae (*e*), ribs (*g*) and hindlimbs (*i,k*). *Sox9* mRNA expression declines in hypertrophic chondrocytes, but β-gal activity was present in these cells, most likely due to the persistence of β-gal protein (*i*). In contrast, in $Sox9^{-/-}$ chimaeras, $Sox9^{-/-}$ β-gal–positive cells were excluded from the corresponding cartilages in the digits (*d*), vertebrae (*f*), ribs (*h*) and hindlimbs (*j,l,m*). Some small patches of $Sox9^{-/-}$ cells (arrows) were observed at the edges of the cartilages (*j,l*), but these cells lacked normal chondrocyte morphology (*j,l,n*). Areas indicated by rectangles in (*l*) are enlarged in (*n,o*).

We generated $Sox9^{+/-}$ and $Sox9^{-/-}$ ES cells (Fig. 1*a–f*). Our targeting strategy functionally inactivated *Sox9* and led to the expression of β-galactosidase (β-gal) activity in a *Sox9*-specific pattern. Because *SOX9* heterozygosity in humans causes postnatal lethality and XY sex reversal, we analysed the behaviour of $Sox9^{+/-}$ and $Sox9^{-/-}$ cells in mouse embryo chimaeras.

We analysed chimaeras at 15.5 days post-coitum (dpc) because most cartilages are well formed at this stage. At 15.5 dpc, the growth plates of the long bones show characteristic zones of resting, proliferating and hypertrophic chondrocytes. Chimaeras derived from $Sox9^{+/-}$ ES cells expressed β-gal activity in a *Sox9*-specific pattern (Fig. 2). In these chimaeras, the various cellular components of growth-plate cartilages appeared normal (Fig. 2*i*). In all cartilages, $Sox9^{+/-}$ cells expressing β-gal had morphology typical of chondrocytes, were present in both resting and proliferative zones of the growth plate and intermingled with wild-type chondrocytes (Fig. 2*i*). In cartilage, the localization of *lacZ* transcripts was identical to those of *Sox9*, *Col2a1*, *Col9a2* and *Agc*, in agreement with our targeting strategy (Fig. 3*a–c*, and data not shown). These results suggest that in chimaeras, $Sox9^{+/-}$ ES cells differentiate into chondrocytes that behave in a manner equivalent to wild-type chondrocytes.

Chimaeras (15.5 dpc) derived from $Sox9^{-/-}$ ES cells were morphologically normal even when composed of a significant proportion (~50%) of $Sox9^{-/-}$ cells. $Sox9^{-/-}$ cells were found to express β-gal in a pattern that was overtly similar to *Sox9* (Fig. 2*b*). Histological examination, however, demonstrated that $Sox9^{-/-}$ β-gal–positive cells were adjacent to, not in, the cartilages (Fig. 2). β-gal–positive $Sox9^{-/-}$ cells appeared to have been physically displaced by wild-type chondrocytes that formed normal cartilages. Moreover, these $Sox9^{-/-}$ β-gal–positive cells had a spindle-shaped mesenchymal appearance (Fig. 2*n,o*), and hence lacked the characteristic morphological features of chondrocytes, which at this stage of embryogenesis are embedded in an abundant layer of extracellular matrix. Occasional $Sox9^{-/-}$ β-gal–positive cells were also found embedded at the periphery of the cartilages, often as single or very small groups of cells (Fig. 2*j,l,n*), but these β-gal–

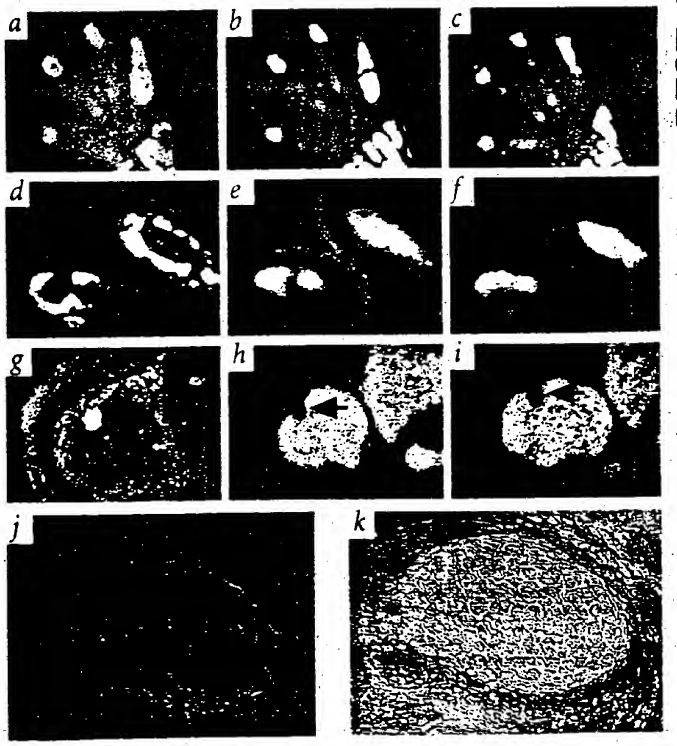

Fig. 3 $Sox9^{+/-}$ (*a–c*) and $Sox9^{-/-}$ chimaeras (*d–k*) at 15.5 dpc. Dark-field illumination of RNA *in situ* hybridization with *lacZ* (*a,d,g*), *Sox9* (*b,e,h*) and *Col2a1* (*c,f,i*) probes. In $Sox9^{+/-}$ chimaeras, *lacZ*, *Sox9* and *Col2a1* transcripts co-localized with each other in the cartilages of the limb (*a–c*). In $Sox9^{-/-}$ chimaeras, *lacZ* transcripts were localized outside of the digit cartilages that expressed *Sox9* and *Col2a1* (*d–f*). *Sox9* and *Col2a1* were not expressed in the small patch of *lacZ*-positive cells (red arrow) embedded in hindlimb cartilage (*g–i*). *j,k*, Double labelling by type II collagen immunofluorescence and X-gal staining. Type II collagen was detected in digit cartilages (*j*), whereas β-gal–positive cells were located surrounding the cartilages (*k*). None of the β-gal–positive cells expressed type II collagen.

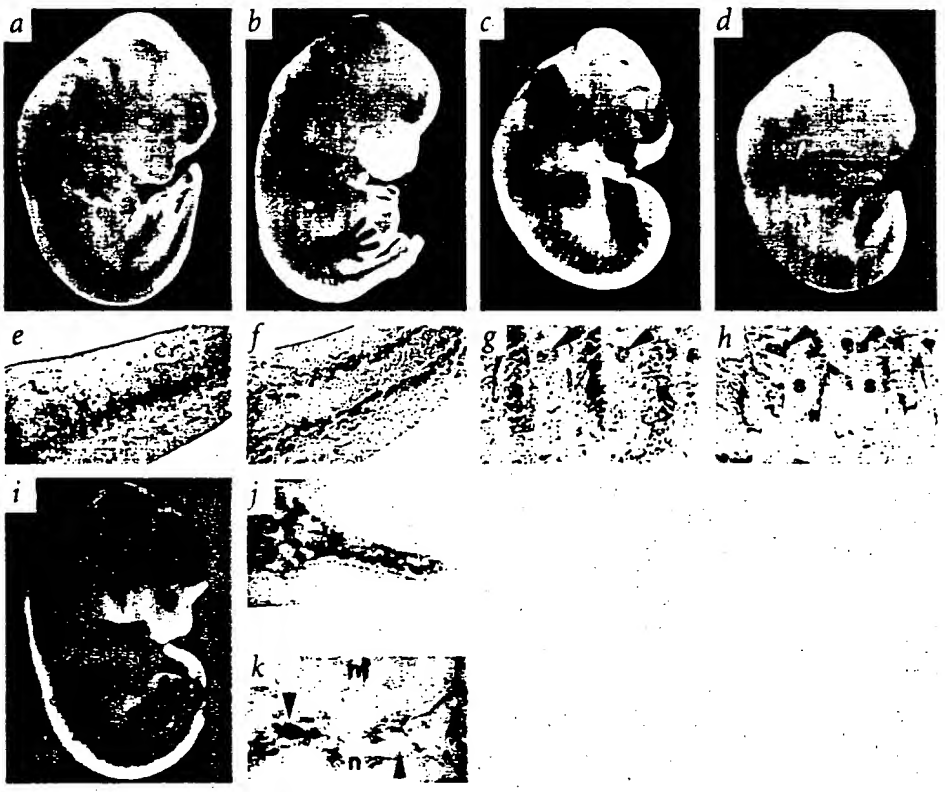

Fig. 4 *Sox9* chimaeras at 11.5 and 12.5 dpc. Whole-mount X–gal stained 12.5 dpc (*a*,*b*) and 11.5 dpc (*c*,*d*) chimaeric embryos derived by injection of $Sox9^{+/-}$ (*a*,*c*) and $Sox9^{-/-}$ (*b*,*d*) ES cells into wild-type blastocysts. Histological analysis of 12.5 dpc $Sox9^{+/-}$ chimaeras showed that $Sox9^{+/-}$ β-gal–positive (blue) cells were located in the mesenchymal condensations of the forelimb buds, intermingled with wild-type (pink) cells (*e*). In contrast, in $Sox9^{-/-}$ chimaeras, $Sox9^{-/-}$ β-gal–positive cells were located adjacent to the wild-type condensing mesenchyme of the forelimb bud (*f*). Histological analysis of 11.5 dpc $Sox9^{+/-}$ chimaeras showed that $Sox9^{+/-}$ β-gal–positive (blue) cells were located within the sclerotomes (s) of the somites, intermingled with wild-type (pink) cells (*g*). In contrast, in $Sox9^{-/-}$ chimaeras, $Sox9^{-/-}$ β-gal–positive cells were segregated from wild-type cells of sclerotomes (*h*). Arrowheads, dorsal root ganglia. *i*, Whole-mount X–gal stained 12.5-dpc chimaeric embryo with a high proportion of $Sox9^{-/-}$ cells. Histological analysis showed that $Sox9^{-/-}$ β-gal–positive cells were present in the precartilage regions of the limb (*j*) and vertebrae (*k*). The large regions of $Sox9^{-/-}$ cells (black arrowhead) appear to disrupt normal morphogenesis. Regions that are predominantly composed of wild-type cells (red arrowhead) are morphologically normal. n, notochord; nt, neural tube.

positive cells did not display normal chondrocyte morphology. The skeletons of these $Sox9^{-/-}$ chimaeras showed no obvious morphological defects and the various cellular layers of the growth plate cartilages were normal and composed entirely of wild-type cells (Fig. 2). *In situ* hybridization indicated that the patterns of *Col2a1*, *Col9a2*, *Agc* and *Sox9* transcripts in the different cartilages overlapped each other, confirming that the cartilages were composed exclusively of wild-type cells (Fig. 3*e*,*f*, and data not shown). In contrast, *lacZ* transcripts were located outside of the cartilages with a pattern of expression distinct from those of *Sox9*, *Col2a1*, *Col9a2* and *Agc* transcripts (Fig. 3*d*). Occasionally, *lacZ* transcripts were present in small patches embedded in wild-type cartilages (Fig. 3*g*); these small patches did not express *Sox9* and *Col2a1* (Fig. 3*h*,*i*). In co-localization experiments, type II collagen was only found in the extracellular matrix surrounding wild-type cells and not around β-gal–positive mutant cells (Fig. 3*j*,*k*). These results suggest that prechondrocytic mesenchyme cells that lack *Sox9* are unable to differentiate into chondrocytes.

To determine when the segregation of $Sox9^{-/-}$ cells from wild-type cells initiated during chondrogenesis, we examined 11.5- and 12.5-dpc chimaeric embryos. At these stages, cartilage primordia consist of condensed mesenchyme cells that are surrounded by much less extracellular matrix; however, these differentiating cells contain abundant *Col2a1* and *Sox9* transcripts. Chimaeras (11.5 and 12.5 dpc) derived from $Sox9^{-/-}$ ES cells had a pattern of β-gal activity in cartilage primordia that mimicked that of *Sox9* expression and that of β-gal activity in the cartilage primordia of chimaeras derived from $Sox9^{+/-}$ ES cells (Fig. 4*a*–*d*). In contrast to $Sox9^{+/-}$ β-gal–positive cells, which were intermixed with wild-type cells in all cartilage primordia (Fig. 4*e*,*g*), $Sox9^{-/-}$ β-gal–positive cells were segregated from wild-type cells (Fig. 4*f*,*h*). Similar to 15.5 dpc chimaeras, the patterns of *Col2a1*, *Sox9*, *Agc* and *Col11a2* transcripts were non-coincident with those of *lacZ*. In contrast, these transcripts were expressed in a similar pattern throughout cartilage primordia (data not shown) of $Sox9^{+/-}$ chimaeras. Thus, even at stages of mesenchymal condensation, $Sox9^{-/-}$ cells did not express chondrocyte differentiation markers and were already segregated from wild-type cells. In earlier stages of chondrogenesis, when prechondrocytic mesenchymal cells do not yet appear as condensations, β-gal–positive cells still appeared intermingled with β-gal–negative cells in caudal sclerotomes and distal parts of limb buds of 11.5 dpc, as well as in sclerotomes of 10.5 dpc chimaeras derived from $Sox9^{-/-}$ cells (data not shown).

We also transplanted $Sox9^{+/-}$ and $Sox9^{-/-}$ ES cell lines into syngeneic mice to generate teratomas. The tumours derived from one wild-type and three $Sox9^{+/-}$ ES cell lines contained many differentiated tissue types, including many cartilaginous structures with typical chondrocytes (Fig. 5*a*,*b*). The chondrocytes in the cartilages of the $Sox9^{+/-}$ teratomas expressed β-gal (Fig. 5*e*). In addition, other differentiated cell types, including mesenchymal and epithelial cells, were also β-gal positive (Fig. 5*f*) because *Sox9* is expressed in many tissue types. The extracellular matrix of wild-type and $Sox9^{+/-}$ cartilages contained type II collagen and expressed *Col9a2* and *Agc* (Fig. 5*h*,*i*, and data not shown). Although teratomas derived from three different $Sox9^{-/-}$ ES cell lines contained many different tissue types, no cartilages, type II collagen, or *Col9a2* or *Agc* transcripts were detected in any tumours (Fig. 5*c*,*g*,*j*, and data not shown). β-gal activity was detected in $Sox9^{-/-}$ tumours, but only in a subset of the non-cartilaginous tissues (Fig. 5*g*). Thus, *Sox9* is essential for cartilage formation in teratomas.

Our chimaera studies suggest that $Sox9^{-/-}$ prechondrocytic mesenchyme cells were initially specified, proliferated and migrated to their proper positions in the body. The subsequent block in the ability of the $Sox9^{-/-}$ cells to differentiate into chondrocytes appears to be at an early stage of chondrogenesis because $Sox9^{-/-}$ cells were already physically segregated from wild-type cells in precartilaginous condensations. Experimental evidence suggests that Sox9 directly activates a set of chondrocyte-specific extracellular matrix genes[11,17–19]. Our results indicate that $Sox9^{-/-}$ cells were unable to express chondrocyte-

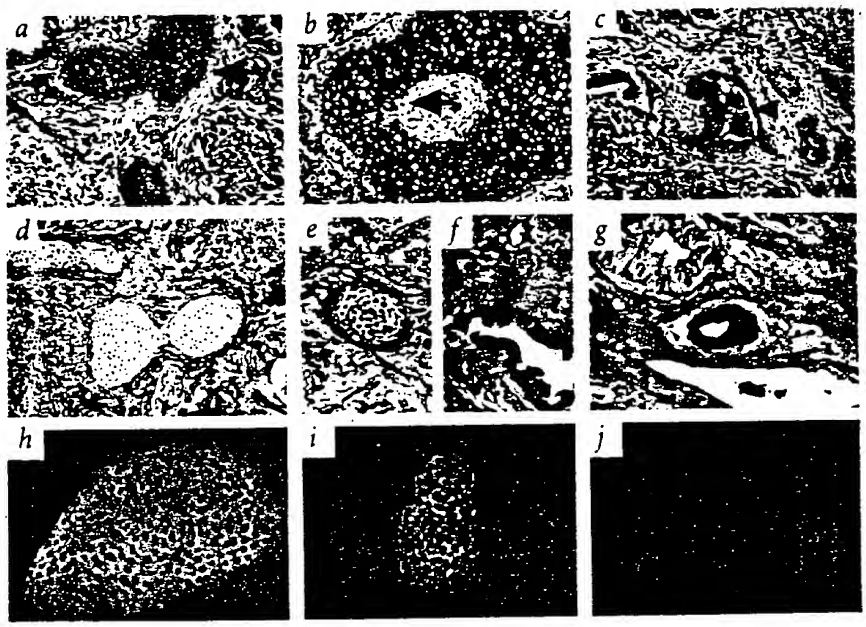

Fig. 5 Teratomas derived from wild-type, *Sox9*$^{+/-}$ and *Sox9*$^{-/-}$ ES cell lines. ***a,d,h***, wild type; ***b,e,f,i***, *Sox9*$^{+/-}$; ***c,g,j***, *Sox9*$^{-/-}$. *a–c*, Alcian blue staining of histological sections. Blue-stained cartilage (arrows) is observed in wild-type and *Sox9*$^{+/-}$ teratomas. Other non-cartilage tissues also stain positively for Alcian blue (arrowheads). *d–g*, β-gal histochemistry. β-gal–positive (blue) cartilage is observed in *Sox9*$^{+/-}$ teratomas (*e*). Other non-cartilage tissues are also β-gal positive (*f*). β-gal–positive tissues are observed in *Sox9*$^{-/-}$ teratomas but none are similar to cartilage (*g*). Type II collagen immunofluorescence (*h–j*). *h,i*, Type II collagen is detected in cartilages of wild-type and *Sox9*$^{+/-}$ teratomas but no immunoreactivity is detected in *Sox9*$^{-/-}$ teratomas (*j*).

specific extracellular matrix genes in chimaeras or teratomas. The segregation of *Sox9*$^{-/-}$ cells from wild-type cells in chimaeras may be due to an inability of *Sox9*$^{-/-}$ mesenchyme cells to interact with the extracellular matrix produced by wild-type condensing mesenchyme before cartilage formation. Perhaps Sox9 also regulates genes encoding cell-adhesion molecules[20,21]. In chimaeras with a very high proportion of *Sox9*$^{-/-}$ cells (>75%), cartilage abnormalities were observed, although chondrocytes in cartilages were exclusively wild type. These skeletal abnormalities may be caused by the interference of a high number of *Sox9*$^{-/-}$ mesenchyme cells and a reduced number of wild-type chondrocytes contributing to the developing skeleton (Fig. 4*i–k*, data not shown). Our studies demonstrate that Sox9 functions cell-autonomously in the formation of cartilages and is an essential transcriptional regulator of chondrocyte cell fate.

Methods

Generation of *Sox9* mutant ES cell lines. The mouse *Sox9* locus was cloned by screening a mouse strain 129/SvEv genomic library using a 300-bp rat *Sox9* cDNA probe. A 4.5-kb *Not*I-*Sac*II 5′ fragment and a 2.5-kb *Sac*I-*Sac*I 3′ fragment derived from the *Sox9* locus were used to generate a targeting vector. An intra-ribosomal entry site (IRES) *lacZ* pA (ref. 22) cassette followed by a *loxP*-flanked PGKneobpA cassette was introduced into the first exon of *Sox9*, simultaneously deleting 450 bp of *Sox9* between *Sac*II and *Sac*I, encoding the translation start site and a portion of the HMG DNA-binding domain. An MC1tkpA herpes simplex virus thymidine kinase expression cassette was added onto the 3′ arm of homology to enrich for homologous recombinants by negative selection with 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU). The targeting vector was linearized at a unique *Not*I site outside of the homology and introduced into AB-1 ES cells as described[23]. G418/FIAU-resistant ES cell clones were initially screened by Southern-blot analysis of *Eco*RI-digested genomic DNA and hybridization with a unique 3′ probe external to the region of vector homology. To reuse G418 selection, the *loxP*-flanked neo cassette was excised from the *Sox9 lacZ*-neo/+ ES cell lines by transient expression of cre recombinase[24]. The neo-deleted *Sox9 lacZ*/+ ES cell clones were identified by Southern blot and verified by G418 selection. The *Sox9 lacZ*/+ ES cell lines were then retargeted with the original vector as described above and verified by Southern blot.

Generation of mouse chimaeras. The *Sox9*$^{+/-}$ and *Sox9*$^{-/-}$ ES cell lines of mouse strain 129/SvEv, which is pigmented, were microinjected into Swiss or CD-1 albino mouse blastocysts to generate chimaeric embryos for analysis between 11.5 and 15.5 dpc. Retinal pigmentation was used to identify chimaeras and to assess the contribution of the ES cells to the tissues of the embryo.

β-gal histochemistry and *in situ* hybridization. Mouse embryos were processed for detection of β-gal activity by X-gal staining[25] as described. Type II collagen was visualized by immunostaining of histological sections as described[18] using an anti-collagen II mouse monoclonal antibody (1:50; DSHB). The secondary antibody was a Texas-red–conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch). RNA *in situ* hybridization was performed as described[26]. Probes for *Col2a1* (ref. 27), *Agc* (ref. 28), *Col9a2* (ref. 29) and *Sox9* (ref. 12) were as described. The *lacZ* probe was a 290-bp *Eco*RV-*Cla*I fragment, and the *Col11a2* probe was a 360-bp fragment from the 5′ UTR.

Generation of teratomas. ES cells (2.0×10^6) were injected subcutaneously into the flanks of 8-week-old 129/SvEv males[30]. The resulting tumours were collected six weeks after transplantation and cut into smaller pieces for β-gal histochemistry. Anti-collagen II rabbit polyclonal antibody (1:100; Rockland) was used for immunofluorescence. The secondary antibody was an FITC-conjugated goat anti-rabbit IgG (1:40; Zymed).

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1. Stockwell, R.A. in *Biology of Cartilage Cells* (Cambridge University Press, Cambridge, UK, 1979).
2. Hall, B. & Newman, S. in *Cartilage: Molecular Aspects* (CRC Press, Boca Raton, Florida, 1991).
3. Mundlos, S. & Olsen, B.R. Heritable diseases of the skeleton. Part I: molecular insights into skeletal development-transcription factors and signaling pathways. *FASEB J.* **11**, 125–132 (1997).
4. Cancedda, R., Descalzi Cancedda, F. & Castagnola, P. Chondrocyte differentiation. *Int. Rev. Cytol.* **159**, 265–358 (1995).
5. Erlebacher, A., Filvaroff, E.H., Gitelman, S.E. & Derynck, R. Toward a molecular understanding of skeletal development. *Cell* **80**, 371–378 (1995).
6. Mundlos, S. & Olsen, B.R. Heritable diseases of the skeleton. Part II: molecular insights into skeletal development-matrix components and their homeostasis. *FASEB J.* **11**, 227–233 (1997).
7. Wagner, T. *et al.* Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the *SRY*-related gene *SOX9*. *Cell* **79**, 1111–1120 (1994).
8. Wright, E. *et al.* The *Sry*-related gene *Sox-9* is expressed during chrondrogenesis in mouse embryos. *Nature Genet.* **9**, 15–20 (1995).
9. Kent, J., Wheatley, S.C., Andrews, J.E., Sinclair, A.H. & Koopman, P. A male-specific role for *SOX9* in vertebrate sex determination. *Development* **122**, 2813–2822 (1996).
10. Morais da Silva, S. *et al. Sox9* expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. *Nature Genet.* **14**, 62–68 (1996).
11. Ng, L.-J. *et al.* Sox9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. *Dev. Biol.* **183**, 108–121 (1997).
12. Zhao, Q., Eberspaecher, H., Lefebvre, V. & de Crombrugghe, B. Parallel expression of *Sox9* and *Col2a1* in cells undergoing chondrogenesis. *Dev. Dyn.* **209**, 377–386 (1997).
13. Hovmoller, M.L. *et al.* Campomelic dwarfism. A genetically determined mesenchymal disorder combined with sex reversal. *Hereditas* **86**, 51–62 (1977).
14. Takahashi, H., Sando, I. & Masutani, H. Temporal bone histopathological findings in campomelic dysplasia. *J. Laryngol. Otol.* **106**, 361–365 (1992).
15. Foster, J.W. *et al.* Campomelic dysplasia and autosomal sex reversal caused by mutations in an *SRY*-related gene. *Nature* **372**, 525–530 (1994).
16. Kwok, C. *et al.* Mutations in *SOX9*, the gene responsible for campomelic dysplasia and autosomal sex reversal. *Am. J. Hum. Genet.* **57**, 1028–1036 (1995).
17. Bell, D.M. *et al.* Sox9 directly regulates the type-II collagen gene. *Nature Genet.* **16**, 174–178 (1997).
18. Lefebvre, V., Huang, W., Harley, V.R., Goodfellow, P.N. & de Crombrugghe, B. SOX9 is a potent activator of the chondrocyte-specific enhancer of the proα1(II) collagen gene. *Mol. Cell. Biol.* **17**, 2336–2346 (1997).
19. Bridgewater, L.C., Lefebvre, V. & de Crombrugghe, B. Chondrocyte-specific enhancer elements in the *Col11a2* gene resemble the *Col2a1* tissue-specific enhancer. *J. Biol. Chem.* **273**, 14998–15006 (1998).
20. Widelitz, R.B., Jiang, T.X., Murray, B.A. & Chuong, C.M. Adhesion molecules in skeletogenesis: II. Neural cell adhesion molecules mediate precartilaginous mesenchymal condensations and enhance chondrogenesis. *J. Cell Physiol.* **156**, 399–411 (1993).
21. Oberlender, S.A. & Tuan, R.S. Expression and functional involvement of N-cadherin in embryonic limb chondrogenesis. *Development* **120**, 177–187 (1994).
22. Mountford, P. *et al.* Dicistronic targeting constructs: reporters and modifiers of mammalian gene expression. *Proc. Natl Acad. Sci. USA* **91**, 4303–4307 (1994).
23. Behringer, R.R., Finegold, M.J. & Cate, R.L. Müllerian-inhibiting substance function during mammalian sexual development. *Cell* **79**, 415–425 (1994).
24. Abuin, A. & Bradley, A. Recycling selectable markers in mouse embryonic stem cells. *Mol. Cell. Biol.* **16**, 1851–1856 (1996).
25. Hogan, B., Beddington, R., Costantini, F. & Lacy, E. in *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1994).
26. Wilkinson, D.G. & Nieto, M.A. Detection of messenger RNA by in situ hybridization to tissue sections and whole-mounts. *Methods Enzymol.* **225**, 361–373 (1993).
27. Metsäranta, M., Toman, D., de Crombrugghe, B. & Vuorio, E. Specific hybridization probes for mouse type I, II, III and IX collagen mRNAs. *Biochim. Biophys. Acta* **1089**, 241–243 (1991).
28. Glumoff, V., Savontaus, M., Vehanen, J. & Vuorio, E. Analysis of aggrecan and tenascin gene expression in mouse skeletal tissues by northern and in situ hybridization using species specific cDNA probes. *Biochim. Biophys. Acta* **1219**, 613–622 (1994).
29. Elima, K. *et al.* Specific hybridization probes for mouse α2(IX) and α1(X) collagen mRNAs. *Biochim. Biophys. Acta* **1130**, 78–80 (1992).
30. Robertson, E.J. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach* (IRL Press, Oxford, UK, 1987).